Isolation of the DNA of Various Plant Pathogenic Mycoplasmalike Organisms from Infected Plants

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ABSTRACT

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DNA from apple proliferation-diseased periwinkle plants and apple trees and from periwinkles infected with mycoplasmalike organisms (MLOs) causing a virescence (chloranty) of rape, a virescence of periwinkle, and a phyllody of *Diplotaxis erucoides* was extracted by using two different methods. Repeated bisbenzimide-CsCl buoyant density gradient centrifugations were used to separate MLO DNA from host plant DNA. MLO DNA was obtained as a well-resolved band showing a lower buoyant density than the host plant DNA due to its low G + C content. This band was highly enriched in MLO DNA and comprised 0.1–3.0% of the total DNA extracted from diseased plants. The DNA of the apple proliferation agent was cloned in *Escherichia coli* and the specificity of the cloned MLO DNA was confirmed by dot and Southern blot hybridization.

Detection of plant pathogenic mycoplasmalike organisms (MLOs) in diseased plants is based mainly on electron and fluorescence microscopy. Both techniques have limited usefulness for several reasons. One of the major disadvantages is that neither technique can differentiate the agents of the respective MLO diseases that affect several hundred plant species (11). Because the organisms can not yet be cultured in vitro, MLOs can only be provisionally differentiated on the basis of symptomatology, host plant range, and pathogen-vector relationships.

Progress has been made in detecting plant-pathogenic MLOs by using serological techniques. Polyclonal antisera have been produced with partially purified MLO antigens from both infected plants (2,3,8,16) and insects (2). However, these antisera showed substantial cross-reaction with antigens from healthy plants. This problem can be overcome by producing monoclonal antibodies against MLO antigens (7). The utility of these highly specific antibodies for characterization and classification of MLOs may be limited because they may be directed against a single MLO epitope. For example, monoclonal antibodies produced against a strain of the aster yellows agent from New Jersey failed to detect the organisms in aster yellows diseased plants from other regions (8).

Further progress has been achieved by developing genomic probes for the detection of the western X-disease MLO (5,6). In this case, the MLO DNA was isolated from infected leafhopper vectors (*Colladonus montanus*) and was cloned in *Escherichia coli*. With these probes the western X-disease MLO could be readily detected in infected plants and insects. No hybridization was obtained with DNA from healthy plants and insects or from *Spiroplasma citri* and several culturable mycoplasmas.

In this paper we report on the isolation of MLO DNA from plants infected with any one of four MLOs including the apple proliferation (AP) agent. AP disease is caused by an MLO whose insect vector is unknown. In such cases, DNA for developing genomic probes can only be obtained by purification from infected plants. Our work on detection of *S. citri* in plants by DNA-DNA hybridization showed that the DNA of the causal agent comprises not more than 0.5% of the total DNA (C. Saillard, *unpublished*). Results on the AP organism were presumed to be similar. In this paper a method is described for isolating MLO DNA from diseased plants. A more detailed description of the cloning of the MLO DNA and the development of specific probes and their application in detection will be published elsewhere.

MATERIALS AND METHODS

DNA sources. DNA was isolated from healthy and AP-diseased apple (*Malus pumila*) trees and periwinkle (*Catharanthus roseus*) plants as well as from periwinkles affected by one of the following diseases: 1) periwinkle virescence, found in 1978 in Lima (Peru) by C. E. Fribourg. 2) Rape (*Brassica napus*) virescence (chloranty), found in southern France by G. Marchoux. 3) Phyllody of *Diplotaxis erucoides*, found by P. Moreno near Valencia (Spain). All diseases except periwinkle virescence were transmitted to periwinkle with *Cuscuta* spp. and were perpetuated on periwinkle by grafting. All diseases transmitted to periwinkle were obtained from R. Marwitz, Berlin.

DNA extraction. Healthy and symptomatic periwinkle plants including roots were frozen at −80 C. In most cases, the leaves were removed. From apple trees (cv. Golden Delicious) phloem of the trunk and the scaffold limbs were sampled in August by removing the bark at cambium level and by separating the translucent layer of the current season's phloem with a knife. The phloem was immediately frozen in liquid nitrogen. The presence of MLOs in diseased trees was confirmed with the DAPI fluorescence test before sampling (15). All material was lyophilized, ground to a fine powder with a hammer mill, and stored at −20 C.

DNA was extracted by using either the modified urea-phosphate-hydroxyapatite (MUP) method (17) or the cetyltrimethylammonium bromide (CTAB) procedure (12), both with some modifications. DNA from healthy and diseased periwinkle was usually extracted by using the CTAB procedure. Thirty grams of tissue powder was suspended in 250 ml of extraction buffer (0.7 M NaCl, 1% CTAB, 50 mM Tris-HCl, 10 mM EDTA, 0.1% dithiothreitol, pH 8.0) and incubated at 55 C for 25 min with occasional gentle mixing. Cell debris, protein, and polysaccharides were removed by extracting the lysate with chloroform:isoamyl alcohol (24:1). After centrifugation, one tenth volume of 0.7 M NaCl and 10% CTAB was added to the aqueous phase after each chloroform extraction, which was repeated three more times.

Then, RNA and DNA were precipitated by adding one volume of 1% CTAB, 50 mM Tris-HCl, and 10 mM EDTA (pH 8.0). After 30 min at room temperature, the nucleic acids were recovered by centrifugation. The pellet was washed three times with 80% ethanol, dried under vacuum, and dissolved in CsCl in 10 mM Tris-HCl, pH 8.0, refractive index 1.4000.

Apple phloem was usually extracted using the MUP procedure. Twenty to forty grams of tissue powder was incubated in 400 ml of MUP buffer (8.0 M urea solubilized in 0.2 M sodium phosphate buffer [PB], pH 6.8, 2.5% SDS) at 37 C for at least 30 min with occasional shaking. Proteins were extracted twice with one volume chloroform:isoamyl alcohol (24:1). The aqueous phase was layered onto a 1-cm thick hydroxyapatite (HAP) (Bio-Rad, Bio-Gel HT) pad, which was formed by pouring 40 ml of HAP equilibrated in three volumes of MUP buffer into a large sintered glass funnel. The lysate was filtered slowly through the HAP pad. When necessary, vacuum and/or gentle stirring was applied to enhance filtration. The binding procedure was repeated twice by passing the filtrate through the funnel. The HAP pad was resuspended in a small volume of MUP buffer and packed in a chromatography column (25 mm diameter). The column was washed with at least 400 ml of MUP buffer until absorption of sample constituents was zero. RNA and ssDNA were eluted at room temperature with 0.2 M PB (pH 6.8) and the dsDNA with a gradient from 0.2 to 0.5 M PB. HAP lots tested by the supplier to elute ssDNA from 0.183 to 0.260 M PB were used under these conditions. The dsDNA was centrifuged at 143,000 *g* for 24 hr at 7 C, and the resulting pellet was resuspended in CsCl as described above.

Separation of MLO DNA. Bisbenzimide-CsCl density gradient centrifugation (10) was used to separate MLO DNA from host plant DNA, which was supposed to have a higher guanine + cytosine (G + C) content than MLO DNA. The method of bisbenzimide (Hoechst 33258; Serva, Heidelberg) binding to DNA was chosen according to the DNA concentration to prevent DNA precipitation. For concentrations up to 50 μg/ml, the bisbenzimide solution (0.5 mg/ml H_2O) was added dropwise under gentle mixing to a dye/DNA ratio of 1.0 (w/w). For higher concentrations up to 150 μg/ml, the bisbenzimide was applied by adding the dye solution into a dialyzing tubing containing a magnetic stirring bar. The sealed tubing was placed into a flask containing the DNA-CsCl solution and was stirred as slowly as possible in the dark for 16 hr at room temperature. The ratio of dye in the tubing and DNA in the flask was 1.0 as above or somewhat higher.

The stained DNA in CsCl solution was adjusted to a refractive index of 1.3940 and centrifuged to equilibrium for 72 hr at 33,000 rpm in a Beckman 50Ti fixed angle rotor at 20 C. The DNA bands were visualized under UV light, and the band containing MLO DNA was removed from the top of the tubes and pooled. Ten microliters of bisbenzimide solution (0.5 mg/ml) per 50 μg of DNA was again added and the DNA was recentrifuged one or two more times for further purification under the same conditions as above. After final centrifugation, the band containing the MLO DNA was extracted three times with one volume of CsCl-saturated isopropyl alcohol to remove the bisbenzimide (14). CsCl was removed by chromatography on Sepharose 4B (Pharmacia LKB) equilibrated with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The DNA was concentrated by ultracentrifugation as described above or by ethanol precipitation. The amount of total DNA was determined spectrophotometrically (260 nm) after the first centrifugation, and that of MLO DNA after the final centrifugation of the upper band. All hybridization and cloning work with MLO DNA was done after the first recentrifugation of the upper band portion of the gradient.

DNA-DNA hybridization. In the dot blot experiments total DNA from healthy and diseased apple trees was denaturated with 50 mM methyl mercurate at room temperature (10 min) and directly spotted onto a nitrocellulose membrane (BA 85, Schleicher and Schuell) using the hybridot apparatus (BRL, Gaithersburg, MD). The membranes were baked at 80 C for 2 hr. Probes were made by nick-translating total, undigested DNA from healthy periwinkle as well as MLO DNA obtained from AP diseased periwinkle using [^{32}P] dCTP (Amersham, specific activity of the probe 2.10 cpm/μg of DNA). Prehybridization was performed at 37 C for 2 hr in 5× SSC (750 mM NaCl, 75 mM sodium citrate), containing 5× Denhart's solution (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin) 50 mM sodium phosphate buffer, pH 6.5, 50% formamide, and 200 μg/ml of denaturated herring sperm DNA. For hybridization, this solution was replaced with a similar solution containing the labeled DNA probe and 10% dextran sulfate. After a 48-hr incubation at 37 C, the filters were washed four times (15 min each time) in a solution of 2× SSC and 1% SDS at room temperature, followed by two washes at 60 C with 0.1× SSC and 0.1% SDS for 2 hr each time. The filters were dried and exposed to X-ray film (Fuji) with an intensifier screen (Lightning Plus, DuPont) for 24 hr at −70 C (1).

For Southern blot analysis, 2 μg of total DNA from both healthy and AP-diseased apple as well as from the MLO band obtained from AP-diseased periwinkle was digested with *Hind*III restriction enzyme (Boehringer Mannheim). The resulting fragments were separated on a 0.7% agarose gel and blotted on a nitrocellulose membrane (BA 85, Schleicher and Schuell). Hybridization with

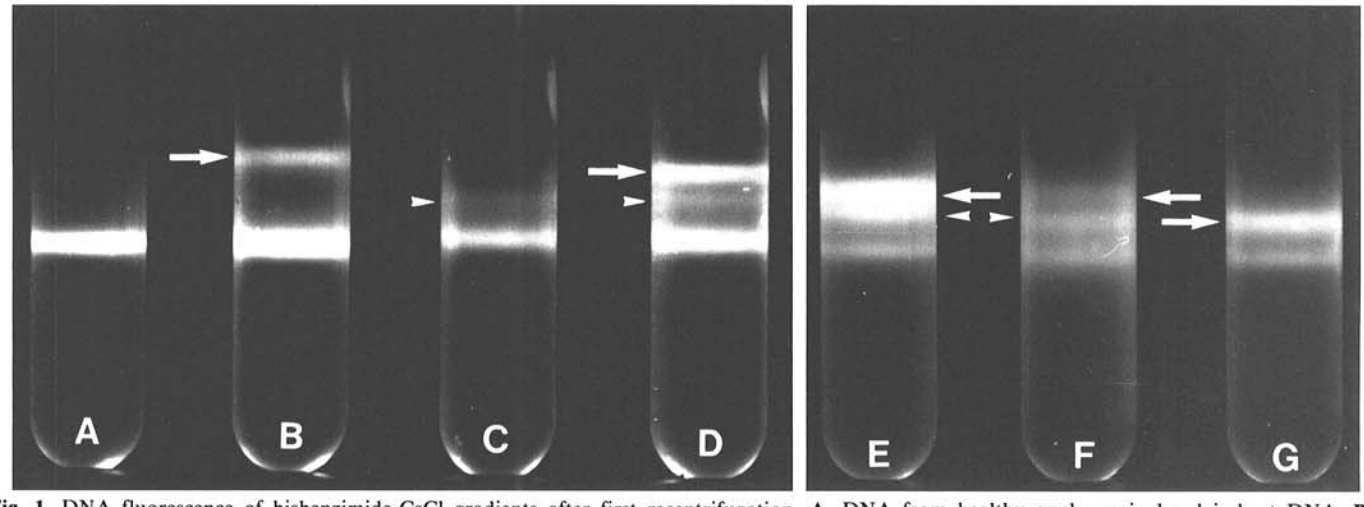

Fig. 1. DNA fluorescence of bisbenzimide-CsCl gradients after first recentrifugation. **A,** DNA from healthy apple, main band is host DNA; **B,** DNA from apple proliferation (AP)-diseased apple, main band and MLO DNA band; **C,** DNA from healthy periwinkle, main band and intermediary band from the host; **D,** DNA from AP-diseased periwinkle, main band, intermediary band, and MLO DNA band; **E,** DNA from rape virescence-diseased periwinkle; **F,** DNA from Catharanthus-virescence-diseased periwinkle; **G,** DNA from Diplotaxis phyllody-diseased periwinkle. The MLO DNA band is marked with an arrow, and the intermediary band of periwinkle with an arrowhead.

nick-translated probes from total DNA of healthy periwinkle and of AP-MLO DNA, as well as autoradiography, were performed as described above (1).

Cloning. Cloning was performed by standard procedures (9). MLO DNA from AP-diseased periwinkle was digested with *Hin*dIII restriction endonuclease (Boehringer Mannheim). Resulting fragments were ligated with *Hin*dIII-digested plasmid pBR322, and were then used to transform *E. coli*, strain HB 101. Recombinant plasmids of ampicillin-resistant strains were extracted and those with inserts larger than 2 kb were identified by agarose gel electrophoresis. These recombinant plasmids were labeled with [^{32}P] dCTP using the random priming technique (Multiprime DNA labeling system, Amersham). They were screened for AP specificity by dot blot hybridization with DNA from both healthy and AP-affected periwinkle plants or apple trees (1).

RESULTS

DNA isolation. The first density gradient centrifugation of DNA from phloem of healthy apple trees revealed one single band with a buoyant density of 1.648 g cm^{-3}. When DNA from diseased apple was centrifuged at a concentration of 250 μg per gradient, an additional faint band with a lower buoyant density was visible. This band became more distinct at higher DNA concentrations. After recentrifugation of the pooled upper band fractions, this band became more prominent, while the host DNA band was strongly reduced. The upper band was located 13 mm above the host DNA band at a buoyant density of 1.620 g cm^{-3} (Figs. 1 and 2A and B). No host DNA band could be detected after the third centrifugation.

The first centrifugation of the DNA from AP-diseased periwinkle plants revealed a main band of host DNA with a BD of 1.641 g cm^{-3}, and an upper band that appeared in correspondence to the applied DNA concentration with the same intensity as described for apple. Recentrifugation of the pooled fractions containing the upper band resulted in three bands: the main band, an intermediary band 7 mm above the main band (BD 1.627 g cm^{-3}), and the upper band 10 mm above the main band (Figs. 1 and 2D). The buoyant density of the upper band was identical to that of the band obtained from diseased apple phloem. If the upper band was carefully removed, no other band was detectable after the third or fourth centrifugation. DNA of healthy periwinkle contained only the main band and the intermediary band (Figs. 1 and 2C). The same patterns were observed with DNA isolated with either the MUP or the CTAB procedure.

MLO DNA bands were also obtained from periwinkle plants infected with the three other MLO diseases investigated (Fig. 1E, F, and G). The BD of the DNA band of the periwinkle virescence agent was similar to that of the AP-MLO. However, the BD of the MLO DNA band of plants affected by rape virescence was slightly higher (1.621 g cm^{-3}) and of those affected by Diplotaxis phyllody was considerably higher than that of the AP-MLO. The DNA band of the Diplotaxis phyllody MLO appeared at about the same position as the intermediary band of periwinkle. The presence of MLO DNA at that position was evidenced by a much stronger fluorescence than in gradients of DNA from healthy plants due to the additional amount of MLO DNA in this band.

The yield of total DNA depended on the extraction method used. From periwinkle, 50–100 μg of DNA per gram of plant powder was obtained with the MUP procedure and about 200 μg with the CTAB procedure. Because the latter method proved to be unsatisfactory for extracting apple phloem, the MUP procedure was applied, which yielded 50–100 μg/g, as in the case of periwinkle. The percentage of total DNA that was MLO DNA was similar for both protocols and the diseases examined but was significantly influenced by the plant parts used for extraction. One tenth to one percent MLO DNA was recovered from periwinkle stems including leaves, about twice as much from stems without leaves, and about three times as much from periwinkle roots. The DNA from diseased apple phloem contained 2% MLO DNA.

DNA-DNA hybridization. Dot and Southern blot analyses were used to prove the MLO nature of the MLO DNA obtained from AP-diseased apple and periwinkle. In the dot blot experiments,

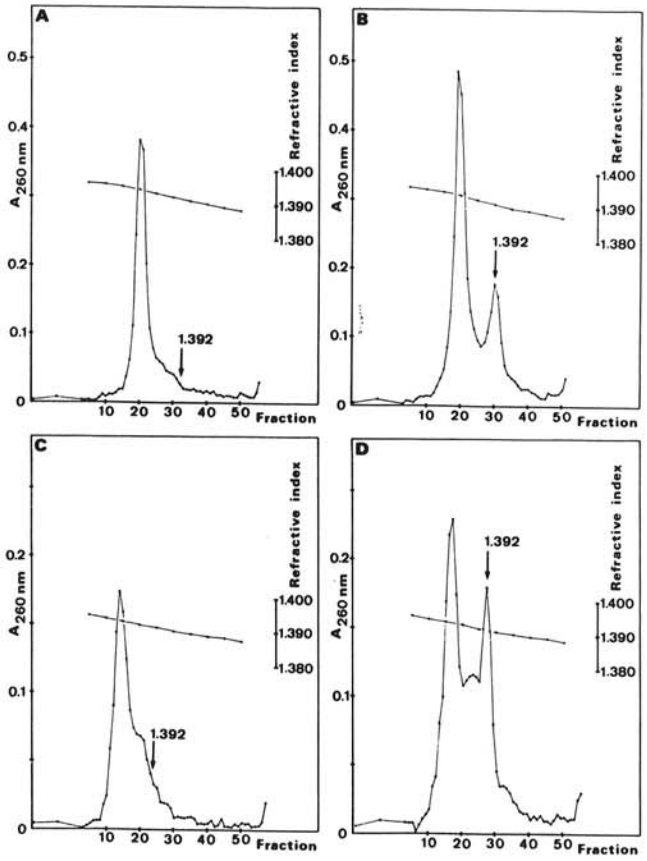

Fig. 2. Elution profiles of the bisbenzimide-CsCl gradients of tubes A–D of Figure 1. DNA from diseased plants shows a peak of apple proliferation-MLO DNA at a refractive index of 1.392 (buoyant density 1.620 g cm^{-3}). The position of the intermediary band of periwinkle can be recognized in C and D as a shoulder or a small peak.

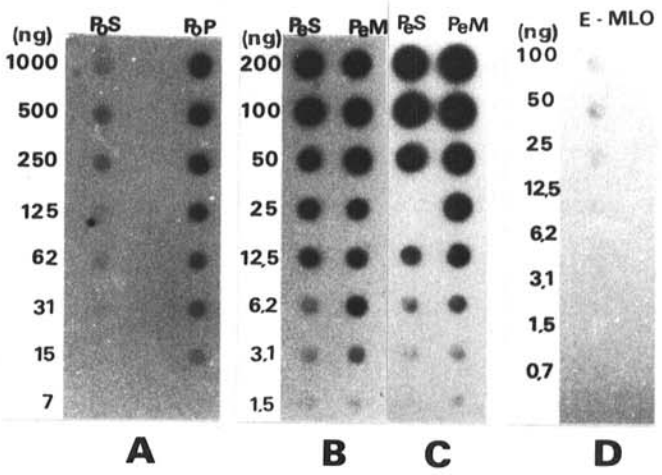

Fig. 3. Dot blot analysis of DNA from healthy and apple proliferation (AP)-diseased apple and periwinkle. **A,** Total DNA from healthy (PoS) and diseased (PoP) apple, hybridized with a probe made from AP-MLO DNA isolated from diseased periwinkle; **B,** total DNA from healthy (PeS) and AP-diseased (PeM) periwinkle hybridized as in A; **C,** total DNA from healthy (PeS) and AP-diseased (PeM) periwinkle hybridized with ^{32}P-nick-translated total DNA from healthy periwinkle; **D,** AP-MLO DNA hybridized as in C.

total DNA from healthy and AP-diseased apple and periwinkle hybridized with the probe made from DNA of AP-diseased periwinkle. However, the probe hybridized much more strongly with the DNA from diseased plants, especially with that from apple (Fig. 3A and B). The reaction of the probe with the DNA from healthy apple indicates that there is some homology between apple and periwinkle. It also shows that the MLO DNA contained some host plant DNA. To eludicate contamination of the MLO DNA band, AP-MLO DNA and DNA from healthy and diseased periwinkle were hybridized with a probe made from DNA of healthy periwinkle. This probe detected 1.5 ng of periwinkle DNA but 25 ng of MLO DNA was necessary to give a signal (Fig. 3C and D). These results show that the major portion of the MLO-DNA band is of MLO origin and that the contamination by host plant DNA is less than 10%.

In Southern blot analysis, the probe made from total DNA of healthy periwinkle hybridized with the DNA from healthy and AP-diseased apple. The homology between these two species is represented by five common bands (Fig. 4A, arrows). When the transferred AP-MLO DNA was hybridized with its homologous undigested probe, 11 distinct bands appeared (Fig. 4B, arrows). All these bands were also recognized in the transferred DNA from diseased apple but none of them in the DNA from healthy apple. The common bands of the digested DNA of apple and periwinkle were also detected with the MLO DNA probe (Fig. 4B, lane 1).

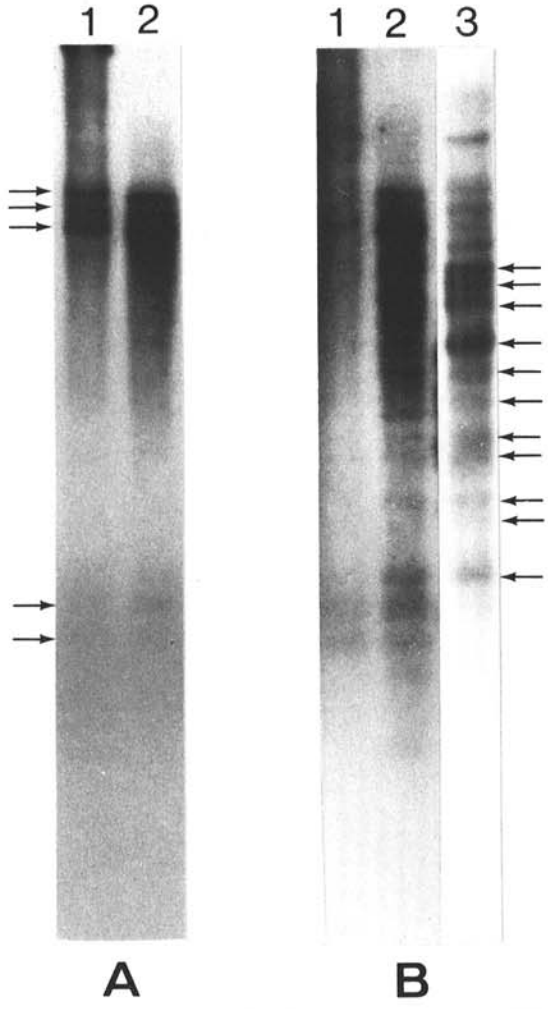

Fig. 4. Southern blot analysis of various DNAs. **A,** Total DNA from healthy (lane 1) and AP-diseased (lane 2) apple digested with *Hin*dIII restriction endonuclease and hybridized with a nick-translated probe made from total DNA of healthy periwinkle; **B,** total DNA from healthy (lane 1) and AP-diseased (lane 2) apple, and MLO DNA band obtained from diseased periwinkle (lane 3) digested as in A and hybridized with a probe of AP-MLO DNA. Arrows indicate MLO-specific bands occurring in the DNA from diseased apple and in MLO DNA.

Specificity of cloned MLO DNA. Nineteen recombinant plasmids with an insert larger than 2 kb were obtained, which in dot blot experiments showed hybridization with DNA from AP-diseased periwinkle plants and apple trees but not with DNA from corresponding healthy plants. They detected the DNA of the AP-MLO in 7–15 ng of total DNA from diseased periwinkle plants and in 15–30 ng from diseased apple trees as shown in Figure 5 for the clone AP184.

DISCUSSION

The upper unique band referred to as MLO DNA band was obtained from plants affected by different diseases and was isolated with two different methods from two plant species. This band was detectable only in extracts from diseased plants and never in those from healthy ones. In dot blot experiments, labeled probes made from undigested MLO DNA obtained from AP-diseased periwinkle hybridized much more strongly with DNA from diseased than from healthy apple. In Southern blot analysis, hybridization of *Hin*dIII-digested DNA from diseased apple with the MLO DNA probe resulted in a profile similar to that obtained by hybridization of *Hin*dIII-digested MLO DNA with its homologous DNA probe. Because none of the common bands occurring in the transferred MLO DNA and the DNA from diseased apple could be observed in the hybridization profile of DNA from healthy apple, these bands must be caused by restriction fragments of MLO DNA. Further evidence for the identity of the MLO DNA was obtained in cloning experiments. The cloned DNA fragments hybridized only with DNA from AP-diseased plants but not with those from corresponding healthy plants. These results together with the findings of other hybridization experiments suggest that the major portion of the MLO DNA band is of MLO origin, although the MLO DNA used in this study was recentrifuged only once. A considerably higher purification

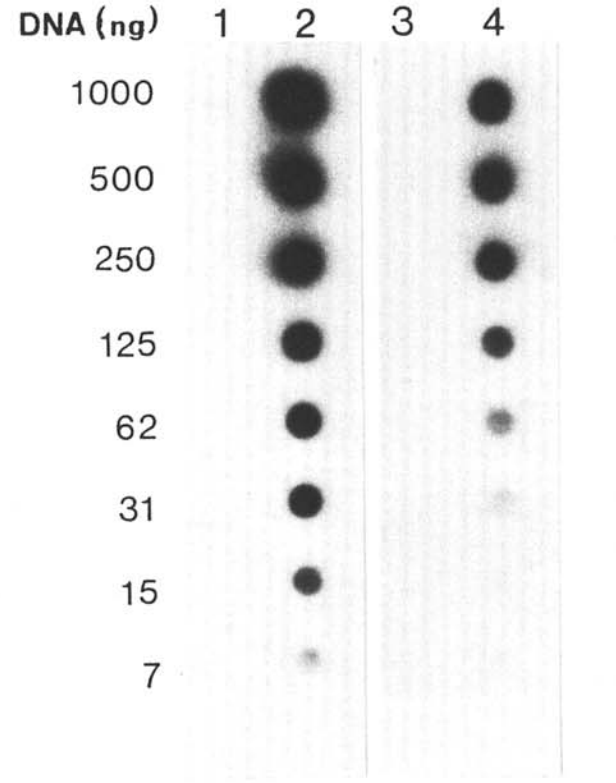

Fig. 5. Dot blot hybridization of various DNAs with a cloned fragment of MLO DNA (probe AP184). **1,** DNA from healthy periwinkle; **2,** DNA from apple proliferation (AP)-diseased periwinkle; **3,** DNA from healthy apple; **4,** DNA from AP-diseased apple.

can be achieved by repeated recentrifugation (A. Kollar, *unpublished*).

The use of the bisbenzimide Hoechst 33258 proved to be very important for the separation of MLO DNA from host plant DNA. This dye binds preferably to A + T-rich sequences, thereby reducing their buoyant density. Dye binding was often used to facilitate separation of DNAs with small differences in the G + C content (e.g., 4,10,13,14). In our case, separation of MLO DNA from the host plant DNA without dye-binding would virtually be impossible due to the low G + C content of apple and periwinkle DNA, and also to the large difference in the relative amounts of plant and MLO DNA, as indicated by an overloaded main band after the first centrifugation step. To obtain substantial quantities of MLO DNA it was necessary to optimize both the bisbenzimide-CsCl gradient and the dye-binding method according to DNA concentration to prevent precipitation of DNA.

Kirkpatrick et al (5) obtained the MLO DNA used for cloning from infected leafhopper vectors. Because for many MLO diseases, including AP, the vectors are not known or not available in sufficient numbers, the DNA has to be isolated from diseased plants. This paper reports on the isolation of highly enriched MLO DNA from plants in considerable quantities. As long as the G + C-content of host plant and pathogen differs sufficiently, DNA of MLOs may be obtained from any MLO diseased plants with the method described in this paper. The availability of MLO DNA to make genomic probes will considerably improve both diagnosis and the demonstration of yet unknown insect vectors of the diseases. Moreover, it will facilitate the differentiation of the numerous MLO diseases and the study of the taxonomic and phylogenetic relationships between various MLOs and between MLOs and other mollicutes.

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